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PRINCIPAL INVESTIGATOR: Xi He, Ph.D.

CONTRACTING ORGANIZATION: Childrens Hospital
Boston, Massachusetts 02115

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6. AUTHOR(S)
Xi He, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Children's Hospital
Boston, Massachusetts 02115

E-MAIL:
xi.he@tch.harvard.edu

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wnt genes encode a large family of secreted signaling molecules essential for development and oncogenesis. wnt-1, the founding member of the wnt gene family, was initially identified as an oncogene. Ectopic wnt-1 expression causes mammary tumorigenesis in mice, providing a potential model for human breast cancer. However, the cell surface receptor (or receptors) that mediates Wnt-1 signaling has not been identified, and the molecular and biochemical nature of the Wnt signaling pathway is not fully understood. In a research supported in part by this Career Development Award, we identified a member of the LDL receptor-related protein (LRP) family, LRP6, as a Wnt-1 co-receptor. We demonstrated that LRP6 binds to Wnt-1, and forms a complex with Fz in response to Wnt-1. Recently, we also identified a potent Wnt-1 signaling antagonist, Dickkopf-1 (Dkk-1), as a ligand for LRP6. These results have important implications in our understanding of Wnt-1 signaling in development and oncogenesis.

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Introduction

The Wnt family of secreted signaling molecules plays essential roles in embryogenesis and tumorigenesis (1). The Frizzled (Fz) family of serpentine receptors has been shown to function as Wnt receptors (2-10), but it remains to be elucidated which Fz specifically mediates Wnt-1 oncogenic function and how Fz proteins transduce Wnt signaling.

The Drosophila *arrow* locus defines a novel segment polarity gene whose mutant phenotype resembles that of the *wingless* (Drosophila Wnt-1) mutation (11). *arrow* encodes a transmembrane receptor (11) homologous to two members of the mammalian low-density lipoprotein receptor (LDLR)-related protein (LRP) family, LRP5 and LRP6 (ref. 12-15). Human LRP6 and LRP5 share 71% amino acid identity, and each contains an extracellular domain with multiple LDLR repeats plus EGF (epidermal growth factor) repeats, followed by a transmembrane domain and a cytoplasmic domain lacking any recognizable catalytic motifs (12-15). A lrp6 gene mutation in mice results in pleiotropic developmental defects, some of which appear to resemble certain Wnt mutant phenotype (16). To study whether/how LRP6 and LRP5 are involved in Wnt signal transduction, including Wnt-1 signaling, we examined the function of LRP6 and LRP5 in Wnt induced secondary axis and neural crest formation in the Xenopus embryo (17). We demonstrated that in two Wnt pathway-dependent developmental processes, secondary axis and neural crest formation, expression of LRP6 activates whereas a dominant negative LRP6 mutant blocks Wnt signaling. These results provide compelling evidence that LRP6 plays a critical role in Wnt signal transduction. Given that LRP6 is a transmembrane receptor-like protein, we are particularly interested in whether it is a Wnt co-receptor.

Body

1). LRP6 forms a complex with Fz in response to Wnt-1

To function as a Wnt co-receptor, LRP6 should bind Wnt or Fz or both. To examine the issue, we utilized a secreted form of mFz8, mFz8CRD-IgG (18), which was composed of the amino-terminal extracellular cysteine-rich domain (CRD) of mFz8 fused with the IgG Fc epitope, and a secreted LRP6N-myc, which consisted of the LRP6 extracellular domain tagged by the myc epitope. mFz8CRD-IgG and LRP6N-myc proteins were incubated together in the presence or absence of Wnt-1. As shown in Figure 1a, mFz8CRD-IgG co-precipitated LRP6N-myc only in the presence of Wnt-1, whereas the secreted IgG fusion partner failed to do so with or without Wnt-1. We performed a reciprocal precipitation using secreted LRP6N-IgG and mFz8CRD-myc, which were generated by swapping the epitope tags. LRP6N-IgG could co-precipitate mFz8CRD-myc, again only in the presence of Wnt-1, whereas the control IgG did not (Figure 1b). Furthermore, LRP6N-IgG also co-precipitated Wnt-1-myc (Figure 1c), a Wnt-1 molecule tagged by the myc epitope. These results suggest that the extracellular domain of LRP6 can bind Wnt-1 and form a complex with Fz in a Wnt-dependent fashion.

2). Wnt antagonist Dickkopf-1 is a ligand for LRP6

While the Fz-LRP6 complex formation induced by Wnt-1 is highly suggestive of a Wnt receptor complex, the significance of this complex in Wnt signal transduction was not

known. Our study on Wnt antagonist Dkk-1 provided a strong support for the co-receptor hypothesis.

Dkk-1 is found from Xenopus to human, and potently inhibits Wnt signaling (19-21). But how Dkk-1 antagonizes Wnt was not known. Dkk-1 binds neither Wnt nor Fz, nor does Dkk-1 affect Wnt-Fz interaction (data not shown). We therefore examined whether Dkk-1 interacts with LRP6. Strikingly, LRP6 extracellular domain, LRP6N-IgG, co-precipitated Dkk1-Flag when the two CM were combined (Figure 2a), whereas neither mFz8CRD-IgG nor the control IgG, each of which was present at a much higher protein level, precipitated Dkk-1 (Figure 2a). Importantly, LDLRN-IgG, the extracellular domain of the related LDL receptor (LDLR) which is not involved in Wnt signaling [30], did not precipitate Dkk-1 despite of its higher abundance than LRP6N (Figure 2a). Conversely, Dkk1-IgG co-precipitated LRP6N-Myc, but not LDLRN-Myc when CM for each protein were mixed (Figure 2b). In fact, Dkk-1 failed to bind to LDLRN even when 20-fold molar excess of LDLRN was present (Figure 2b), demonstrating again a high selectivity of Dkk-1-LRP6 interaction. Using Dkk1-AP and LRP6N-IgG in a liquid phase enzyme-linked binding assay, we demonstrated that Dkk-1-LRP6 interaction was specific and saturable, and exhibited an affinity constant (Kd) of 0.5 nM using binary binding Scatchard analysis (Figure 2c).

3). Dkk-1 disrupts Fz-LRP6 complex formation induced by Wnt-1

Our previous experiments have shown a complex formation between the extracellular domains of Fz8 and LRP6 in the presence of Wnt-1 (Figure 1). Strikingly, Fz8-LRP6 complex induced by Wnt-1 was completely abolished by Dkk-1, that is, mFz8CRD-IgG no longer associated with LRP6N-Myc in a Wnt1-dependent fashion when either Dkk1-Flag or Dkk1-AP or Dkk1-IgG, but not the control CM, was present (Figure 3a and data not shown). The ability of Dkk-1 to disrupt Fz8-LRP6 complex appeared very potent, as the disruption occurred even when Dkk-1 in the CM was deluted to a barely detectable level (Figure 3a), likely reflecting the high affinity interaction observed between Dkk-1 and LRP6. It was shown that LRP6N exhibits Wnt binding activity (Figure 1c). Although Dkk-1 did not affect Wnt-1 binding to Fz (data not shown), Dkk-1 inhibited Wnt-1 binding to LRP6N (Figure 3b), suggesting that Wnt-1 and Dkk-1 binding to LRP6 is mutually exclusive. We also found that LRP5N, the extracellular domain of LRP5 protein which is functionally and structurally related to LRP6, could also associate with mFz8CRD in a Wnt-1 dependent fashion, and this complex formation was abolished by Dkk-1 as well (Figure 3c).

Conclusions

The Wnt family of secreted growth factors initiates signaling via the Frizzled (Fz) receptor and its candidate co-receptor, LDL receptor-related protein 6 (LRP6), most likely through Wnt-induced Fz-LRP6 complex formation induced by Wnt. Dkk-1 is a high affinity ligand for LRP6, and inhibits Wnt signaling by preventing Fz-LRP6

complex formation that is induced by Wnt.. Our findings suggest that Wnt-Fz-LRP6 complex formation, but not Wnt-Fz interaction, triggers Wnt signal transduction.

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Figure legends

Figure 1. LRP6 extracellular domain binds Wnt-1 and complexes with mFz8CRD in the presence of Wnt-1.

(**a**) mFz8CRD-IgG co-precipitated LRP6N-myc in the presence of Wnt-1. All components in the precipitation reaction were provided via conditioned medium (CM). mFz8CRD-IgG or the control IgG was incubated with LRP6N-myc in the presence of Wnt-1, control CM, or fresh medium. The amount of input mFz8CRD-IgG or the control

IgG in CM mixtures was determined by anti-human IgG antisera (Panel I), and that of LRP6N-myc by an anti-myc antibody (Panel III). mFz8CRD-IgG or the control IgG in mixtures was precipitated with Protein G beads, and precipitates were immunoblotted with the anti-myc antibody to detect LRP6N-myc (panel IV), or with the anti-human IgG antisera to monitor efficacy of precipitation (panel II).

(**b**) LRP6N-IgG co-precipitated mFz8CRD-myc in the presence of Wnt-1. Procedures were identical as to (**a**), except that epitope tags were reciprocated.

(**c**) LRP6N-IgG co-precipitated Wnt-1-myc. Although LRP6N-IgG was precipitated less effectively than the control IgG (Panel II, compare with Panel I), significantly more amount of Wnt-1-myc was co-precipitated with LRP6N-IgG (Panel IV). Under the same conditions used, mFz8CRD-IgG co-precipitated Wnt-1-myc more effectively (not shown). Results in (**a-c**) were repeated at least three times. * indicates IgG from bovine serum.

Figure 2. Dkk-1 is a high affinity ligand for LRP6

(a) Dkk1-Flag co-precipitated with LRP6N-IgG, but not mFz8CRD-IgG or LDLRN-IgG. 1 ml of Dkk1-Flag was mixed with 0.2 ml of IgG (lane 2), 0.2 ml of mFz8CRD-IgG (lane 3), 1 ml of LRP6N-IgG (lane 4), or 1 ml of LDLRN-IgG (lane 5). Protein G precipitates (IP) (I, III, V) and CM mixtures before precipitation (II, IV, VI) were immunoblotted with anti-Flag (I, II) or hIgG (III to VI) Abs. Background bands in panels e and f probably reflect aberrant IgG fusion proteins from pRK5 based vectors.

(b) LRP6N-Myc, but not LDLRN-Myc, co-precipitated with Dkk1-IgG. 0.5 ml of Dkk1-IgG was mixed with either 0.5 ml of control CM (lane 1), or 0.5 ml of LRP6N-Myc (lane

2), or 0.5 ml of LDLRN-Myc (lane 3), or 0.25 ml of each LRP6N-Myc plus LDLRN-Myc (lane 4). Protein G precipitates (IP) (I, III) or CM mixtures before precipitation (II, IV) were immunoblotted with anti-Myc (I, II) or hIgG (III, IV) Abs.

(c) Dkk1-AP binding to LRP6N-IgG (circles, line) or IgG (squares). The horizontal axis shows Dkk1-AP concentration (nM), the vertical axis shows changes in absorbency at 405 nm per hour. Insert is the binding data presented in Scatchard plot.

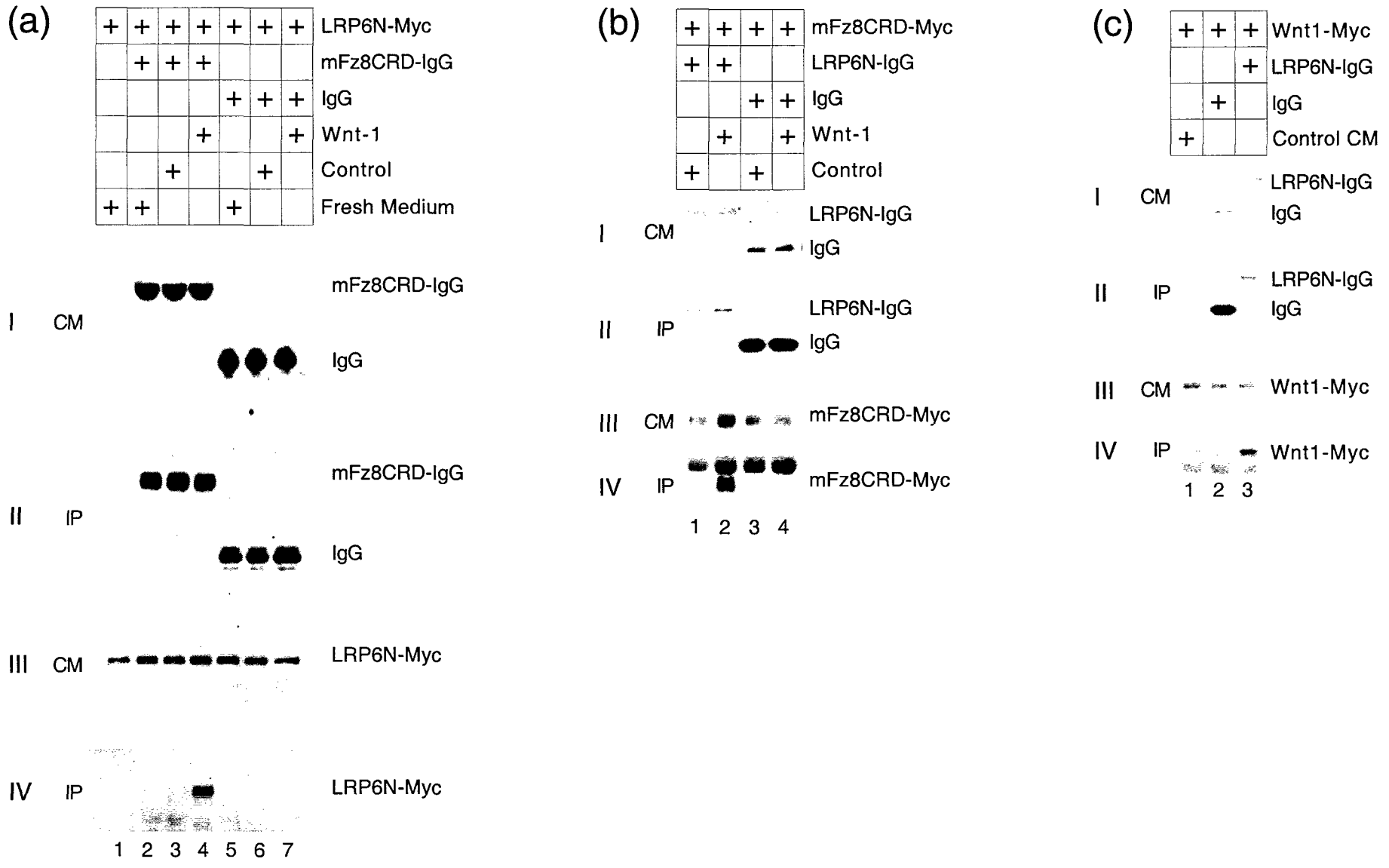
Figure 3. Dkk-1 blocks Fz-LRP5/6 complex formation induced by Wnt-1

(a) Dkk1-Flag prevents Wnt-1 induced mFz8CRD-LRP6N complex formation. 0.6 ml of LRP6N-Myc and 0.25 ml of mFz8CRD-IgG were mixed without or with 1 ml of Wnt-1 (lanes 1 and 2), or with 1 ml of Wnt-1 plus 2 ml or 0.1 ml of Dkk-1-Flag (lanes 3 and 4) or corresponding volumes of control CM (lanes 5 and 6). Protein G precipitates (IP) (I, III, V) and CM mixtures before precipitation (II, IV, VI) were immunoblotted with anti-Myc (I, II), Flag (III, IV), or hIgG (V, VI) Abs. Note the effectiveness of Dkk1-Flag CM even when it was deluted to a barely detectable level (lane 4).

(b) Dkk1-Flag inhibits Wnt-1-LRP6N interaction. 5 ml of LRP6N-IgG (lanes 1 and 2), control IgG (lane 3), or control CM (lane 4) were incubated with protein G beads, followed by incubation with 1.5 ml Dkk1-Flag and 1.5 ml Wnt1-Myc (lane 1) or 1.5 ml control CM and 1.5 ml Wnt1-Myc (lanes 2 to 4). Protein G precipitates (IP) (I, III, V, VI) and CM mixtures (II, IV) were immunoblotted with anti-Myc (I, II), Flag (III, IV), or hIgG (V, VI) Abs. For detection of LRP6N-IgG (about 200 Kd) and the control IgG (35 Kd), the same sample was divided into two parts which were separated by 5.5% and 12% gels, respectively. Equal exposures to X-ray films were applied.

(c) Dkk1-Flag prevents Wnt-1 induced mFz8CRD-LRP5N complex formation. 1 ml of LRP5N-Myc and 0.7ml of mFz8CRD-IgG were mixed without (lane 1) or with 1 ml of Wnt-1 (lane 2), or with 1 ml of Wnt-1 plus 0.5 ml of Dkk1-Flag (lane 3). Protein G precipitates (IP) (I, III) and CM mixtures before precipitation (II, IV) were immunoblotted with anti-Myc (I, II) or hIgG (III, IV) antibodies.

Figure 1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Wnt-1
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II IP
mFz8CRD-IgG
IgG
III CM
LRP6N-Myc
IV IP
LRP6N-Myc
1 2 3 4 5 6 7
(b)
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LRP6N-IgG
IgG
Wnt-1
Control
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IgG
II IP
LRP6N-IgG
IgG
III CM
mFz8CRD-Myc
IV IP
mFz8CRD-Myc
1 2 3 4
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LRP6N-IgG
IgG
Control CM
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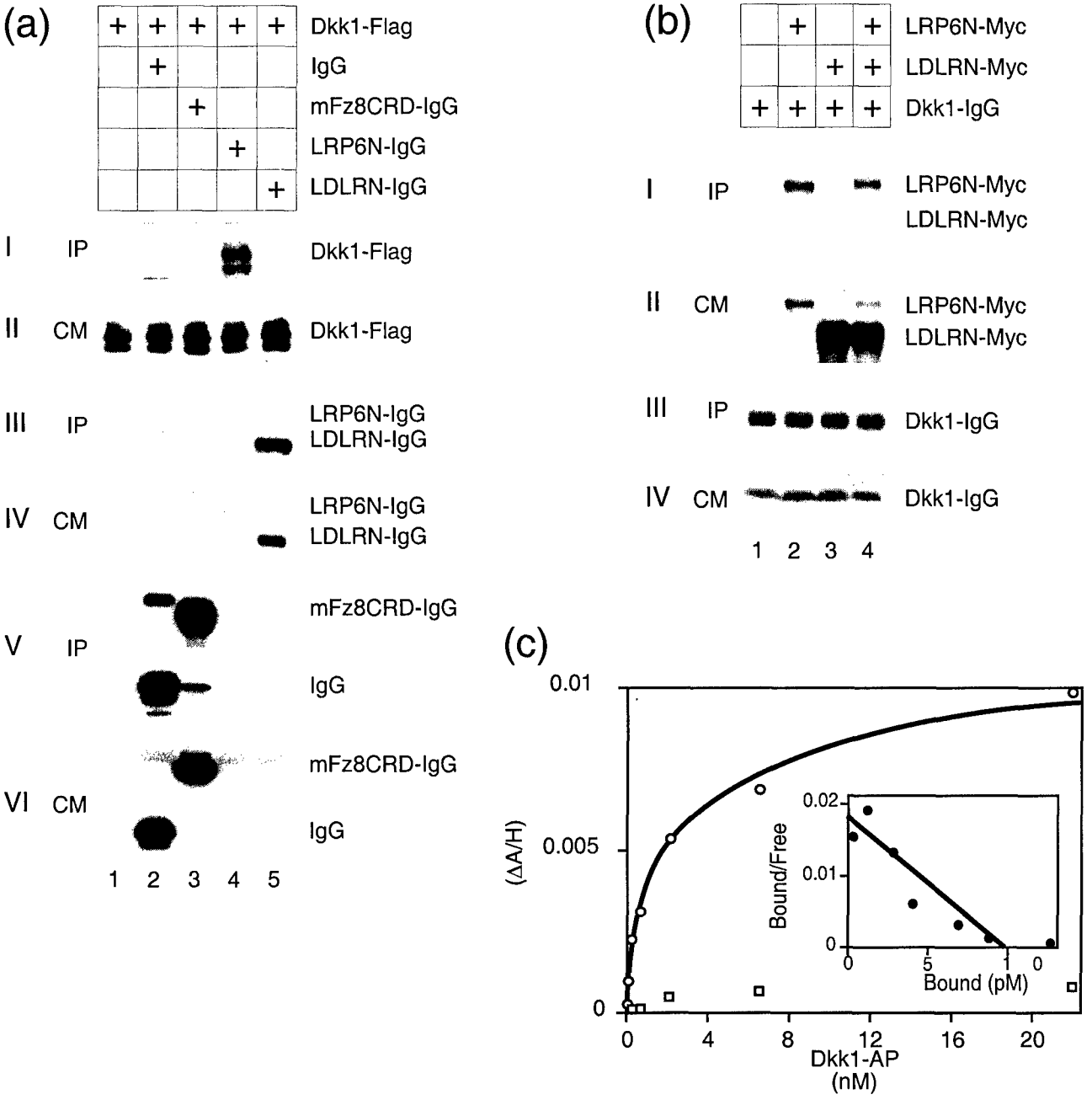
Wnt1-Myc
1 2 3

Figure 2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LRP6N-IgG
LDLRN-IgG
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LDLRN-IgG
V IP
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IgG
VI CM
mFz8CRD-IgG
IgG
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Bound/Free
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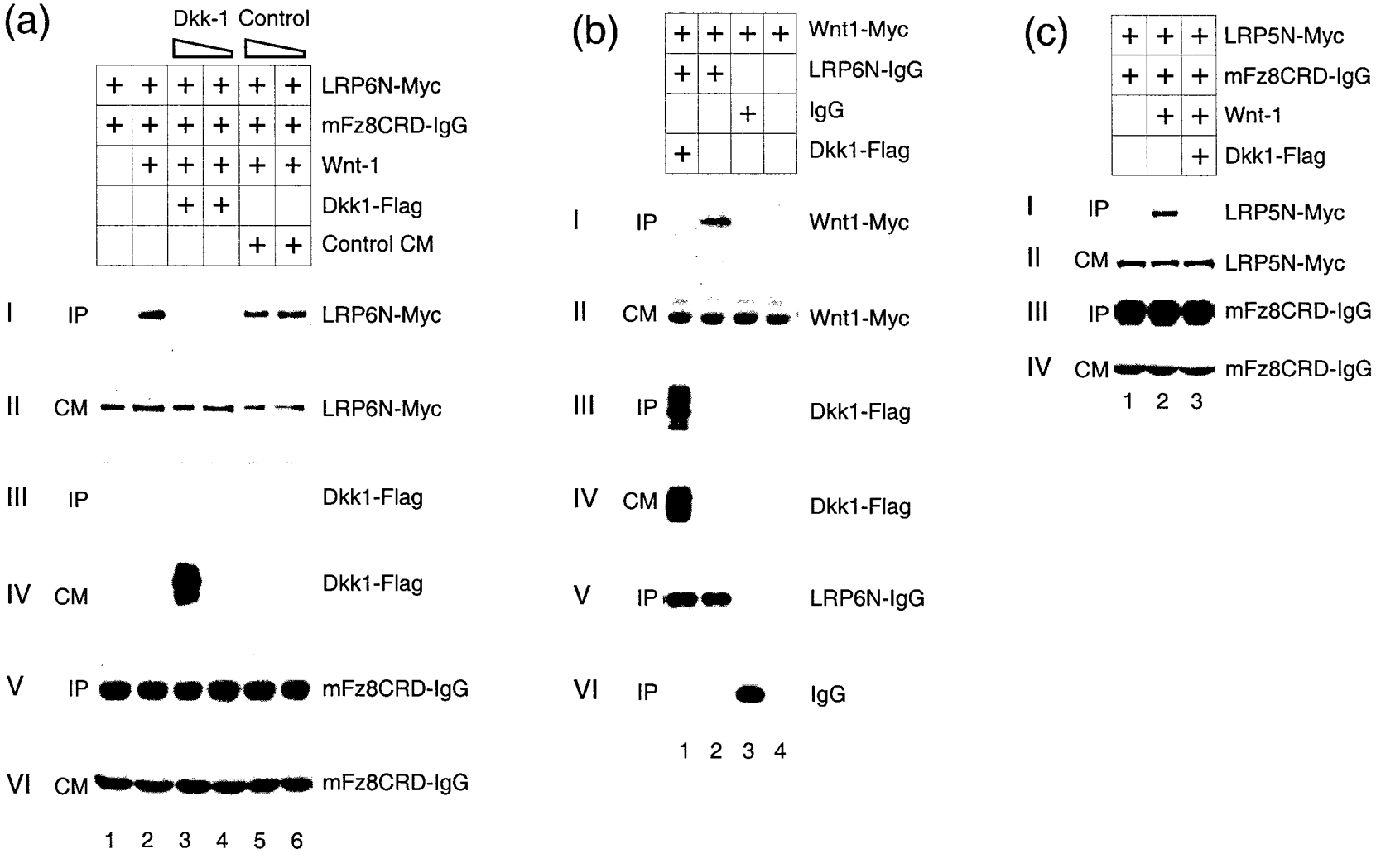
Bound (pM)

Figure 3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III IP Dkk1-Flag
IV CM Dkk1-Flag
V IP mFz8CRD-IgG
VI CM mFz8CRD-IgG
1 2 3 4 5 6
(b)
Wnt1-Myc
LRP6N-IgG
IgG
Dkk1-Flag
I IP Wnt1-Myc
II CM Wnt1-Myc
III IP Dkk1-Flag
IV CM Dkk1-Flag
V IP LRP6N-IgG
VI IP IgG
1 2 3 4
(c)
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mFz8CRD-IgG
Wnt-1
Dkk1-Flag
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II CM LRP5N-Myc
III IP mFz8CRD-IgG
IV CM mFz8CRD-IgG
1 2 3